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LITERATURE REVIEW OF DNA-BASED SUBSPECIES ANALYSIS OF BACILLUS ANTHRACIS, BURKHOLDERIA PSEUDOMALLEI, BURKHOLDERIA MALLEI, AND YERSINIA PESTIS

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The identification of DNA-based polymorphisms can be a powerful means for the subspecies identification of bacterial species. In the case of pathogens, the kind of discrimination can enable the source of an outbreak to be tracked with a certain degree of precision. The likelihood of strain identity correlates with the number of polymorphisms detected. Published references of DNA-based ploymorphisms were reviewed for four human pathogens; Bacillus anthracis, Burkholderia pseudomallei, Burkholderia mallei, and Yersinia pestis. Considerable research has been accomplished for the identification of polymorphisms from the strains B. anthracis and B. pseudomallei. The B. anthracis literature includes several cases in which molecular approaches were used for the analysis of the source of the Sverdlovsk, USSR, outbreak in 1979. No references were found describing any subspecies DNA-polymorphisms for B. mallei, and only relatively few references were found describing the identification of such polymorphisms from Y. pestis.

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Preface

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CONTENTS

5

LITERATURE REVIEW OF DNA-BASED SUBSPECIES ANALYSIS OF *BACILLUS ANTHRACIS, BURKHOLDERIA PSEUDOMALLEI, BURKHOLDERIA MALLEI,* AND *YERSINIA PESTIS*

1. INTRODUCTION

In recent years, the scientific basis for the identification of microorganisms has undergone a shift in emphasis from the traditional reliance on biochemical and microscopic identification of phenotypic characteristics to new techniques based on nucleotide sequence heterogeneities[1]. The DNA-based technologies provide an extremely stable and reproducible means for discriminating organisms at the genus and species level. In addition, for some organisms, these techniques can and have been used to distinguish strains at the subspecies level, providing a sound basis for the epidemiological tracking of the likely source of an outbreak. The identification of DNA polymorphisms has also permitted the design of primers for the Polymerase Chain Reaction (PCR)[2] based detection of microorganisms from clinical and environmental samples. Although potentially applicable to any organism, this technology has been particularly applied to pathogens due to their impact on public health.

This review focuses on DNA-based technologies used to identify the bacteria *Bacillus anthracis, Burkholderia mallei, Burkholderia pseudomallei* and *Yersinia pestis.* These organisms offer a significant contrast with respect to the work accomplished on the subspecies identification of each. In two of these cases (*B. anthracis* and *B. pseudomallei*), a number of strains have been identified by either ribotyping or PCR-based methods. In the case of *Y. pestis*, only a few studies have been conducted, although their results are illuminating. In the case of *B. mallei*, essentially none of this type work has been published as of 1999. However, classical bacterial genetics and molecular biology efforts with the *B. mallei* organism have led to the construction of a number of different antibiotic-resistant strains of this organism. These efforts suggest the need for an effective means of tracking *B. mallei* strains at the subspecies level.

2. DISCUSSION

2.1 Overview of Applicable Techniques.

A number of different techniques can be used to produce diagnostic DNA banding patterns, visible either directly from a gel or via autoradiography. The number of bands may vary from around 5-12 (ribotyping) up to several dozen or more for the different PCR-based techniques. The techniques are generally complementary, since they are based on different sequences or means of detection. Any variable region of DNA could potentially serve as a polymorphic marker Most techniques for the detection

of these polymorphisms are based on either Southern hybridization[3] or PCR. Several of the most frequently utilized techniques are briefly described below.

2.1.1 Ribotyping.

Bacterial ribosomal RNA (rRNA) operons exist as gene families with highly conserved sequences. For instance, rRNA from *Escherichia coli* will hybridize to the rRNA genes of many, if not all, other bacterial DNA. Bacterial chromosomal DNA is digested with restriction endonucleases (typically those such as *Bam*H1, *Pst*I or *Eco*R1 that tend to cut only outside the rRNA operons) and size fractionated on an agarose gel. The polymorphisms in the flanking restriction sites cause fragment size changes (restriction fragment length polymorphisms, or RFLPs) of the DNA fragments containing the rRNA genes. These fragments are typically visualized using chemiluminescent or radioactively labeled DNA probes followed by autoradiography. The ribotyping technique offers the advantages of relative ease of performance and ease of interpretation. In addition, the procedure can be automated using a RiboPrinter®[4], although the resolution its less than that obtained with manual gel systems, as shown using *Vibrio cholerae* O1 DNA[5]. Sixteen clinical isolates from four different countries were analyzed with the RiboPrinter® system and by the traditional method using agarose gel electrophoresis, followed by transfer of the size-fractionated DNA fragments to a nylon membrane and probing with digoxigenin-labeled cDNA prepared from *E. coli* 16S and 23S rRNA. The RiboPrinter® system produced only five different *Eco*RI ribotypes compared to 10 with the traditional system. By automated ribotyping, 12 of 16 strains were designated as the same ribotype, while manual ribotyping divided that same group into six types.

2.1.2 Random Amplified Polymorphic DNA (RAPD).

RAPD is essentially PCR with arbitrary primers, typically around 10 bases in length. The random sequences bind to a number of homologous sequences found in a given genome and permit amplification of the intervening sequences. Generally, two low-stringency PCR cycles are followed by high stringency PCR with specific primers. The resulting banding patterns can be diagnostic for particular strains if the primed region spans a polymorphic sequence.

2.1.3 Amplified Fragment Length Polymorphism (AFLP).

This technique was originally developed for use in plants[6,7] and has now been applied to human, animal and bacterial DNA[8] as well. It essentially combines RFLP and PCR methodology and no prior sequence knowledge is required. Genomic DNA is digested with restriction enzymes and primer-specific adapters are ligated to the fragments. The corresponding primers are added and the amplified fragments are analyzed by size-fractionation on a gel. Typically, 30-80 fragments are amplified per reaction.

2.1.4   Analysis of Repetitive DNA: Variable Number of Tandem Repeats (VNTR) or Short Sequence Repeats (SSR) Regions.

Repetitive DNA consists mainly of homopolymeric [poly(A), poly(T), poly(G) or poly(C)] or multimeric (short sequence) repeats. Until recently, repetitive DNA was primarily known to exist in eukaryotic organisms, where they have proven very useful for DNA fingerprinting[9]. More recently, these repetitive sequences have been described in prokaryotic organisms as well. Belkum et al. have provided a review of prokaryotic repetitive DNA[10]. It is clear that these repetitive sequences offer the potential to identify a large number of polymorphisms, even within otherwise highly monomorphic species. Less clear yet though, is the extent of correlation between these phenotypes and these highly variable genotypes.

2.1.5   Pulsed Field Gel Electrophoresis (PFGE).

Certain restriction enzymes (NotI, SfiI, SmaI, etc.) cut at sites with long recognition sequences and therefore produce only a small number of relatively large restriction fragments. The resulting fragments can be resolved on an agarose gel using PFGE, which uses pulsed electrical fields to cause the DNA fragments to migrate primarily lengthwise through the gel matrix. The resulting size fractionation pattern is often diagnostic for a particular species or strain.

2.2   Review of DNA-Based Subspecies Analysis of Four Pathogens.

2.2.1   *Bacillus anthracis*.

B. anthracis is a gram positive organism, the causative agent of anthrax in a number of mammalian species including cattle and humans., *B. anthracis* is a remarkably monomorphic species, differentiated primarily on the basis of the presence or absence of two plasmids, pXO1 and pXO2, both of which are required for pathogenesis in humans. Consequently, these plasmid sequences serve as a basis of discrimination between virulent and avirulent strains. Ramisse et al. developed a multiplex PCR assay for characterization of *B. anthracis* isolates, and simultaneous confirmation of the species independent of plasmid content[11]. Their assay amplifies the *lef, cya* and *pag* genes from pXO1 and the *cap* gene from pXO2, as well as a *B. anthracis* specific chromosomal marker. Their system unambiguously identified virulent (pXO1$^+$/pXO2$^+$) and avirulent (pXO1$^+$/pXO2$^-$, pXO1$^-$/pXO2$^+$ and pXO1$^-$/pXO2$^-$) strains of *B. anthracis* and distinguished "anthrax-like" strains from other *B. cereus* strains.

2.2.1.1   PFGE Analysis of *B. anthracis* DNA.

PFGE provides a means to distinguish *B. anthracis* strains from *B. cereus* from *B. mycoides* using the enzymes NotI, SfiI, and SmaI. However, this approach does not distinguish *B. anthracis* strains Ames, Vollum and Sterne from each other[12].

9

2.2.1.2 PCR of the 16S-23S Intergenic Spacer Region of B. anthracis.

Harrell et al. and Bourque et al. amplified the 143-144 bp 16S-23S rDNA intergenic spacer region (ISR) and found a polymorphism capable of distinguishing B. anthracis, B. cereus, B. mycoides and B. thuringiensis from each other[12,13]. However, B. anthracis Ames, Sterne and Vollum had identical sequences in this region and were therefore indistinguishable.

2.2.1.3 PCR of the B. anthracis Gyrase Gene ISR.

Harrell et al. also amplified the ISR of the gyrase gene and found a polymorphism capable of distinguishing B. anthracis from B. cereus from B. mycoides[12]. Again, the same polymorphism was not capable of distinguishing B. anthracis Ames, Vollum and Sterne.

2.2.1.4 PCR of the cap Region of B. anthracis.

Makino et al. used a 288 bp PCR product from the cap region as a probe in Southern hybridizations[14]. This approach distinguished B. anthracis, B. cereus, B. licheniformis, B. megaterium, B. subtilis, and B. thuringiensis from each other as well as from other non-Bacillus bacteria. It was sensitive (detecting as little as one sporeforming unit) and practical (capA sequences of B. anthracis were detected in mouse blood and spleen samples within 8 hours of administration of B. anthracis). However, the assay did not discriminate between B. anthracis Ames, Vollum and Sterne.

2.2.1.5 Analysis of AFLP Patterns in B. anthracis DNA.

The AFLP approach was used by Keim et al. to screen 79 different B. anthracis strains from most regions of the world[15]. This approach was notable in that it provided the ability to screen a large number of strains across a relatively large percentage of the B. anthracis genome (ca. 6.3% of the genome was examined for length mutations and ca. 0.36% was examined for point mutations). They detected 31 polymorphic chromosomal regions from an analysis of 357 AFLP polymorphic fragments among the 79 isolates. A total of 1221 fragments were observed for polymorphisms; 97% were monomorphic for all 79 strains. About half the AFLP markers were length variants (VNTRs). The AFLP technique also proved very useful for elucidating the Bacillus genus taxonomy. The authors concluded "the ongoing anthrax epidemic in Canada and the northern United States is due to a single strain introduction that has remained stable over at least 30 years and a 1,000 mile distribution."

2.2.1.6   Analysis of VNTR Patterns in *B. anthracis* DNA.

Andersen *et al.* were the first to find VNTRs in B. anthracis[16]. Two alleles were noted and consisted of two or four consecutive repeats of CAATATCAACAA. This sequence was found in the open reading frame (ORF) designated *vrrA* or variable region with repetitive sequence. It was identified using arbitrarily primed PCR with a primer sequence from the M13 phage. The Sterne and Vollum strains were identical over the 1,480-bp genomic DNA fragment except that the Sterne strain contained four consecutive repeats of CAATATCAACAA whereas the Vollum strain had two of these repeats deleted. The Ames strain differed from Sterne by a single-nucleotide deletion.

Jackson *et al.* expanded the VNTR work with their PCR analysis of 198 *B. anthracis* strains[17]. This effort revealed 5 polymorphisms consisting of 2-6 copies of the *vrrA* sequence CAATATCAACAA. Based on the observed frequency of the five different alleles, polymorphisms in this site should be capable of discriminating between 60% of future *B. anthracis* isolates. The different alleles corresponded to the geographical distribution of the respective strains and all 198 pure cultures contained only one allele each. Additionally, multiple colonies from single infections contained only one allele each, a finding consistent with each infection originating from a single organism. The authors noted that this kind of diversity within an ORF suggests that the variation might be functional; perhaps related to antigenic variation.

VNTRs were subsequently used to analyze tissue samples from 11 victims of the 1979 Sverdlovsk, USSR anthrax outbreak[18]. High quality DNA was extracted and amplified using double PCR with nested primers. Results demonstrated that the entire complement of *B. anthracis* toxin and capsular antigen genes required for pathogenesis were present in tissues from all the victims. Analysis of *vrrA* sequences demonstrated that at least four of the five known strain categories were present, suggesting the source of the infection was a mixture of at least four strains. These findings were significant in light of the disagreements over the source of the outbreak. Western governments claim it resulted from an accidental release from a nearby military microbiology facility whereas USSR officials attributed it to consumption of contaminated meat.

2.2.1.7   Analysis of a Variable Region of the *B. anthracis* Protective Antigen Gene.

Price *et al.* identified six different haploid types from among 26 divergent *B. anthracis* strains by sequencing the protective antigen gene (*pag*) of each[19]. The protective antigen is the portion of the *B. anthracis* toxin that binds to the eukaryotic cell surface receptors and mediates transport of the toxins into the cell. Sequencing data revealed five point mutations, three synonymous and two missense (all transitions), corresponding to six haploid types comprising three different amino acid sequences. Two of the synonymous mutations occurred only once; the others were present with frequencies ranging from 3/26 to 20/26. The amino acid changes were located near a highly antigenic region critical to lethal factor binding. The authors also used nested

11

primers to amplify and sequence a 307-bp variable region of *pag* from necropsy samples from ten Sverdlovsk anthrax victims. From these samples, a total of five different alleles were recovered, corroborating the results of Jackson *et al.* (1998), in which it was shown that at least four different *vrrA* genotypes were present. The five *pag* genotypes included two not seen in the 26-samples survey. Both were transition mutations, one synonymous, the other a novel missence mutation located again just adjacent to the highly antigenic region of PA domains 3 and 4. Phylogenetic analysis of the *pag* suggested that plasmid evolution in *B. anthracis* has occurred with little or no horizontal transfer between strains. Some of the Sverdlovsk samples also showed evidence of infection by multiple strains, again corroborating the results of Jackson *et al.*[17].

2.2.2   *Burkholderia mallei.*

B. *mallei* was previously known as *Pseudomonas mallei, Malleomyces mallei,* and *Actinobacillus mallei.* The bacterium is a Gram-negative rod and an obligate parasite. It is the causative agent of glanders in horses, donkeys and mules and can also infect humans, cats, dogs and many other mammals under experimental conditions. Hamsters or mice are the most common laboratory models. Mortality is very high, there is no vaccine, and antibiotics are typically only partially effective, which sometimes leads to latent infections with the organism sequestered in abscesses in organs. Glanders has disappeared from most regions of the world, leaving only enzotic foci in Asia and eastern Mediterranean countries, and sporadic human cases among those whose occupations involve direct contact with infected equines or work in laboratories.

No references were found to any work with *B. mallei* DNA polymorphisms for the purposes of subspecies identification. However, there has been some research identifying DNA sequences useful for the discrimination of *B. mallei* from other species including the closely related *B. pseudomallei.* Also, molecular genetics and classical bacterial genetics studies on this organism have been published, mainly in Russian journals. Although somewhat peripheral to the main focus of this review, a brief discussion of those studies is included below.

2.2.2.1   Antibiotic Resistance in *B. mallei* Strains.

Batmanov determined the antibiotic sensitivity for eight different *B. mallei* strains[20]. Minocycline, doxycycline, methacycline and chlortetracycline were tested in experimentally infected golden hamsters. Minocycline was the most efficient antibiotic, followed by doxycycline, chlortetracycline and methacycline. The latter was described as inefficient.

Ilyukhin *et al.* tested several antibiotics in experimentally infected golden hamsters[21]. For the treatment of animals infected subcutaneously with *B. mallei,* ofloxacin proved to be most effective, followed by biseptol, doxycycline and

minocycline. None of the antibiotics tested were effective against 160 LD$_{50}$ of *B. mallei* aerosol. Only one antibiotic (doxycycline) was 70% effective against 16 LD$_{50}$ of *B. mallei* aerosol.

Stepanshin *et al.* did a correlation of the frequency of mutations determining resistance to five different antibiotics and "the influence of the resistance mutations on the culture virulence"[22].

2.2.2.2   Transfer of pTH10a from *E. coli* to *B. mallei*.

Ageeva *et al.* transferred the plasmid pTH10a from *E. coli* KS707 into six wild strains of *P. mallei* (Ts-4, 8, Ts-5, 11, Budapest and Ivanovich[23]. The pTH10 plasmid was stably maintained in *B. mallei* even without the selective pressure of antibiotics. The transconjugants simultaneously inherited tetracycline, kanamycin and ampicillin resistance genes. Tetracycline resistance was increased from 0.1-0.5 ug/ml to 50-200 µg/ml, kanamycin resistance was increased from 0.5-1.0 µg/ml to 250-500 µg/ml, and ampicillin resistance was increased from 50-250 µg/ml to 500-2000 µg/ml. The conjugal function of the plasmid was also retained, permitting transfer of the antibiotic resistance genes between *B. mallei* strains with the same frequency as from *E. coli* KS707 (pTH10).

2.2.2.3   Transfer of the Broad Host Range Vector RSF 1010 into *B. mallei*.

Anishchenko *et al.* transferred the broad host range vector RSF1010 into *B. mallei* and *B. pseudomallei* with the aid of the conjugative plasmids pTH10 and RP1[24]. The authors also constructed two RSF1010/pBR322 derivatives called pVA1 and pVA4 (13.1 and 11.5 kb, respectively), which were also transferred into *B. mallei* and *B. pseudomallei*. Plasmids pVA1 and pVA4 were stably maintained in *B. mallei* and *B. pseudomallei* and are potential cloning vectors for use in these organisms.

2.2.2.4   Development of *B. mallei* and *B. pseudomallei* Cloning Vectors.

Abaev *et al.* successfully introduced several recombinant plasmids into *B. mallei* via conjugation[25]. The plasmids were stably maintained, even after a single passage through the bodies of golden hamsters. They found that *B. mallei* strain Ts-5 was almost as efficient a recipient strain as *Escherichia coli* HB101. Three cloning vectors (pES154, pES161 and pSM525) were developed for *B. mallei* and *B. pseudomallei*. Although gene expression levels were low in the *E. coli* host, the vectors proved very effective for cloning *B. mallei* and *B. pseudomallei* genes into *Pseudomonas cepacia.*

2.2.2.5   Development of *E. coli* Libraries of *B. mallei* and *B. pseudomallei* Genes.

Abaev *et al.* successfully constructed *B. mallei* and *B. pseudomallei* libraries in the *E. coli* recipient HB101[26]. These libraries were found to express *B. mallei*

and *B. pseudomallei* antigenic determinants, suggesting the possibility of development of a vaccine from cloned proteins.

2.2.2.6   PCR Discrimination of *B. mallei* and *B. pseudomallei*.

Bauernfeind *et al.* developed a PCR procedure for the detection and discrimination of *B. mallei* and *B. pseudomallei* based on a single nucleotide difference in the 23S rDNA (T versus C at position 2143)[27]. Three different *B. mallei* strains were tested but all three had thymidine at position 2143 and could not therefore be distinguished from each other on this basis.

2.2.3   *Burkholderia pseudomallei*.

B. pseudomallei is a Gram-negative rod found in the soil in Southeast Asia and Northern Australia. It is the causative agent of melioidosis, typically infecting humans through lesions in the skin. There is no vaccine available and the organism is often found to be antibiotic resistant, apparently due at least in part to the presence of a multidrug efflux system (AmrAB-OprA), which is specific for both aminoglycoside and macrolide antibiotics[28]. Rapid diagnosis is essential; more than half the patients die within the first two days after hospital admission, before the cultures turn positive. Because the melioidosis is a significant public health problem, *B. pseudomallei* is well-characterized with respect to subspecies analysis of DNA polymorphisms.

2.2.3.1   Ribotyping of *B. pseudomallei*.

B. pseudomallei is amenable to ribotyping; a total of at least 22 different ribotypes have been identified by a number of different investigators[29-35].

2.2.3.2   RAPD Analysis of *B. pseudomallei* Clinical Isolates.

Haase *et al.* used RAPD analysis for the subspecies identification of 27 clinical isolates of *B. pseudomallei*[36]. The purpose of the investigation was to determine whether melioidosis relapse cases were most likely the result of re-emergence of the initial infecting strain or re-infection with a different strain. The results were consistent with the majority of relapse cases being caused by re-emergence of the initial infecting strain. The authors also demonstrated that RAPD analysis was more discriminating than ribotyping for the subspecies identification of *B. pseudomallei*.

2.2.3.3   Comparison of RAPD and Multilocus Enzyme Electrophoresis.

Norton *et al.* analyzed 18 clinical isolates by RAPD and multilocus enzyme electrophoresis (MEE)[37]. The RAPD and MEE results were found to be consistent, although RAPD was more discriminating, separating two isolates found to be identical by MEE. The study was initially undertaken in order to investigate a possible epidemiological link between melioidosis cases occurring at the same time in

four different regions in North Queensland. However, it was found that the isolates clustered genotypically on the basis of disease presentation, rather than geographical location. The results suggested a bacterial genetic basis for a particular manifestation of melioidosis (i.e. respiratory disease vs. encephalitis vs. visceral abscesses).

2.2.3.4   PCR Techniques for Rapid Clinical Detection of B. pseudomallei.

Several investigators have developed PCR techniques for the rapid clinical detection of B. pseudomallei[38-40]. One PCR technique detects as little as 1 cell/ml blood[41]. In the case of septicemic melioidosis, rapid detection of the organism from blood is essential due to the rapid progression of clinical symptoms.

2.2.3.5   Identification of Insertion Sequences in B. pseudomallei.

Mack and Titball, using primers designed from the nucleotide sequences of five insertion elements from Burkholderia cepacia, found that insertion sequences (IS) homologous to those found in virulent B. cepacia were present in virulent B. pseudomallei but not in avirulent B. pseudomallei[42]. This was the first demonstration of mobile genetic elements in B. pseudomallei. The authors hypothesized that these mobile elements may be an important source of genetic diversity for B. pseudomallei, either by direct insertional inactivation or by increasing the level of expression of genes downstream of their insertion site via the effect of their own promoter.

2.2.3.6   Analysis of B. pseudomallei Flagellin Gene Sequences.

Another source of DNA polymorphisms was found by Winstanley et al.[43] They reported variation in the flagellin sequence among four environmental isolates of B. pseudomallei but not among six clinical isolates of the same organism. These polymorphisms were found to be complementary to PFGE.

2.2.3.7   XbaI/PFGE Polymorphisms of B. pseudomallei Clinical Isolates.

Vadivelu et al. conducted PFGE separations on XbaI digests of 49 clinical isolates of B. pseudomallei and identified a number of different strains within different ribotypes[44]. This complementarity of the PFGE and ribotyping was important since a relatively large number of isolates tend to fall into only 3 of the 22 known ribotypes.

2.2.4   Yersinia pestis.

Y. pestis is a Gram-negative coccobacillus, the causative agent of bubonic and pneumonic plague. The organism is transferred to humans from fleas carried by rats. Rapid progression and high lethality characterize the disease. Three well-documented plague pandemics have occurred, beginning in the 6th century (Africa and Mediterranean countries), 14th through 18th century (Europe) and the most recent which began 1894 in Hong Kong and rapidly spread over five continents, due to

modern transportation. Historically, probably hundreds of millions of people died, especially in the first two pandemics. Although plague is currently controllable with antibiotics and although there is a vaccine available for individuals at high risk, the number of human cases has recently been increasing[45], leading to its categorization as a reemerging disease.[46]

2.2.4.1   _Y. pestis_ Ribotyping.

In 1994, Guiyoule _et al._ conducted ribotype analysis of 70 strains isolated from five continents over 72 years.[47] They found a total of 16 _Eco_RI and _Eco_RV ribotypes, two of which (B and O) characterized 65.7% of the strains. The other 14 types were found in no more than three strains each. Great heterogeneity was found among the strains from Africa, relatively less heterogeneity was found among the Asian isolates and the American strains were of a single ribotype. The authors noted a correlation between ribotypes and existing biovars and determined that the ribotypes were stable after five passages _in vivo_. They established a clear correlation between the ribotypes and the history of the three plague pandemics.

In 1997, Guiyoule _et al._ expanded their previous study of _Y. pestis_ ribotypes with an examination of 187 strains isolated from Madagascar from 1939 to 1996.[48] Isolates were responsible primarily for human cases of bubonic and pneumonic plague. They found that all strains isolated before 1982 were of the classical ribotype B, the same ribotype that spread around the world during the third pandemic. In 1982, 1983 and 1994, new ribotypes (R, Q and T) were isolated in Madagascar. A follow-up study indicated these strains had become well-established and were supplanting the classical B ribotype strain.

2.2.4.2   PFGE Analysis of _Y. pestis_ DNA.

PFGE is generally problematic with _Y. pestis_ analysis, due to a genomic instability, believed to originate from a common 120 kb deletion. Guiyoule _et al._ found that different colonies from the same strain displayed different PFGE patterns.[47] However, Rakin and Heeseman demonstrated in 1995 that PFGE of I-_Ceu_I restriction digests can be used to distinguish the three _Y. pestis_ biovars.[49] In addition, pigmented vs. non-pigmented strains could be distinguished by using PFGE analysis of _Bin_/I digests.

2.2.4.3   PCR-Based Detection and Discrimination of Yersinia Strains.

Trebesius _et al._ used PCR methodology to differentiate _Y. pestis_ and three different strains of the closely-related _Y. pseudotuberculosis_ based on one nucleotide difference in a highly variable region of the 23S rDNA.[50] The procedure detected as few as 100 _Y. pestis_ cells per ml with seminested PCR. The procedure was also used to differentiate _Y. enterocolitica_ biogroup 1B from biogroups 2 to 5, but not for subspecies identification of _Y. pestis_ strains.

2.2.4.4   _Y. pestis_ Highly Repetitive Sequences.

Mozharov _et al._ reported finding highly repetitive sequences (HRS) in _Y. pestis_.[51] The HRS sequences were cloned and used as probes of DNA from 120 _Y. pestis_ isolates. When used as probes of strains not belonging to the genus _Yersinia_, the probes did not hybridize. About 10% of the strains of _Y. pseudotuberculosis_ hybridized to HRS (serovars 1 and 3 only) and were found to contain between one and four copies of HRS. However, the HRS homology was apparently weak, as the probes did not hybridize under stringent conditions (temperatures above 73°). No HRS sequences were found in _Y. enterocolitica_. All 120 strains of _Y. pestis_ contained a substantial number of copies of HRS elements. One to two copies of HRS were found on the pestinogenicity plasmid (pPst) and three to eight copies were found on the plasmid determining the synthesis of the antigen of fraction I and "mouse" toxin (pFra/Tox). The pFra/Tox HRS copies also occurred in different locations on the plasmids. HRS elements were not detected in the calcium dependent (pCaD) plasmids isolated from _Y. pestis_ 352 and _Y. pestis_ 231. The authors noted that the genomic HRS elements were stable with respect to location and copy number, despite intensive laboratory manipulations which led to the loss of from one to three plasmids and changes in virulence. In their survey of 120 _Y. pestis_ strains isolated from the former USSR, Mongolia, Vietnam and Northeast China from human patients, rodents and fleas between 1975 and 1990, the authors found notable correlations between HRS patterns and the origin of the strains. Correlation was also found between HRS patterns and levels of expression of fraction I antigen from its plasmid, suggesting a possible regulatory role for HRS. Finally, it was noted that an HRS pattern change (addition of one supplementary HRS locus) in strains isolated between April, 1990 and May, 1990 resulted in and LD_{50} dose almost 50 times greater that that of the strains isolated in April 1990.

3.   SUMMARY

B. anthracis is a remarkably monomorphic species, exhibiting relatively few DNA polymorphisms useful for the subspecies discrimination. This is probably due to its lifecycle as a pathogen, in which it is continually subjected to the selective pressures of pathogenesis, requiring it to successfully colonize immunocompetent hosts. Animals offer a rather narrow ecological niche, as compared to soil, for instance. Therefore, pathogens can be very tightly adapted to that role, resulting in relative genetic homogeneity. Nevertheless, both AFLPs and VNTRs have proven very useful for subspecies analysis of _B. anthracis_ and large population studies have been completed on _B. anthracis_ strains using these techniques. In addition, subspecies identifications have been used to track the likely source of outbreaks. For instance, Keim _et al._ determined that based on AFLP marker similarity, the ongoing anthrax epidemic in Canada and the northern United States is probably due to a single strain introduction that has remained stable over at least 30 years and a 1,000 mile distribution[15]. Another example (DISCUSSION, above) is the use of _B. anthracis_ VNTR

and *pag* sequences to track the Sverdlovsk, USSR outbreak to a source of at least four different VNTR strain categories or five different *pag* categories. Since one strain exhibits only one VNTR or *pag* genotype, the implication of those investigations is that the victims were infected by a *B. anthracis* source comprised of at least five distinct strains. That finding is seemingly inconsistent with the explanation of the outbreak as being the result of the consumption of contaminated meat. Another interesting finding of those studies was that some of the victims had apparently been infected with more than one strain, as more than one VNTR and/or *pag* sequence was detected. It was previously believed that infections were clonal; i.e., the result of a single bacterium infecting the host.

For *B. mallei*, no DNA-based subspecies identification references were found. However, PCR-based methods have been developed to distinguish *B. mallei* from the closely-related *B. pseudomallei*. Also, significant progress has been made with the introduction of antibiotic resistant genes into *B. mallei* strains and with the use of *B. mallei* as a transduction recipient. *B. mallei* is almost as efficient a transduction recipient as *E. coli* HB101 and several different antibiotic resistant plasmids have been successfully introduced to the species and even maintained without the need for antibiotic selection[23]. Libraries of both *B. mallei* and *B. pseudomallei* have been completed in an *E. coli* host, using broad host-range cloning vectors.

Significant progress has been made with the DNA-based subspecies analysis of *B. pseudomallei*, primarily due to its impact on public health in Southeast Asia and Northern Australia. Ribotyping, RAPD, IS, PFGE and Flagellin gene polymorphisms have all proven useful, and in at least some cases, complementary to each other for the subspeciation of *B. pseudomallei*. Given the overall similarities of *B. pseudomallei* and *B. mallei*, these same techniques may offer the means for subspecies identification of *B. mallei* as well.

Y. pestis does exhibit polymorphisms with respect to ribotypes, although most strains isolated have the same ribotype, so the technique's usefulness has thus far been limited. PFGE also offers some potential for subspecies discrimination, although interpretation of the results can be complicated by genomic instability. HRS sequences apparently provide for considerable diversity, which in at least one case corresponded to an almost 50-fold change in the LD_{50}. However, the details of investigations into HRS sequences are not yet available in English.

In conclusion, the four bacteria *B. anthracis, B. mallei, B. pseudomallei* and *Y. pestis* offer considerable contrast with respect to the extent of knowledge about their subspecies identification. *B. anthracis* has been the subject of considerable recent focus in the field of molecular biology, resulting in the identification of a number of successful PCR-based approaches to its subspecies identification. *B. pseudomallei*, which comprises a significant public health hazard in Southeast Asia and Northern Australia, has been extensively identified by several different approaches, including ribotyping. *B. mallei* offers a contrast with the first two organisms in that no published

works were found with respect to any type of subspecies identification of the organism. Finally, *Y. pestis*, which offers a more localized public health threat than *B. pseudomallei*, has been subjected to only limited subspecies identification efforts.

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